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The 60-kDa Heat Shock Protein (HSP60) of the Sea Anemone *Anemonia viridis:* A Potential Early Warning System for Environmental Changes

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Abstract: Expression of heat shock proteins (HSPs) is often correlated with adaptation to environmental stress. We examined the role of HSP60 (60 kDa) in acclimatization to thermal stress in the sea anemone *Anemonia viridis.* Using monoclonal antibodies, we identified HSP60 in sea anemones for the first time, and showed that its expression varied with changes in seawater temperature (SWT). *Anemonia viridis* displayed high levels of HSP60 when extreme temperatures prevailed in stressful habitats such as tidal pools. Specimens sampled from different temperature layers in the same tidal pool differed in their levels of HSP60. Specimens from subtidal zones exhibited a seasonal pattern of expression of HSP60, according to the seasonal SWT. The level of HSP60 was significantly higher in the summer (SWT, 31°C) than in other seasons throughout the year. This study suggests the use of HSP60 expression as a tool for stress detection in marine invertebrates.

Key words: heat shock proteins, environmental stress, acclimatization, HSP60, Mediterranean, *Anemonia viridis,* immunoblotting, early warning system.

INTRODUCTION

The adaptation of organisms to broad fluctuations in the abiotic conditions of their habitat has always posed an interesting ecological and evolutionary problem. Extreme or unexpected short-term or long-term changes in the environment can be regarded as stressful for organisms (Brown and Howard, 1985; Sanders, 1993), whose ability to respond seems to be crucial in their adaptation to highly fluctuating environments. Over the course of evolution, organisms have developed a capacity to withstand environmental stress by means of several mechanisms, such as behavioral adaptations, morphological changes, regulation of reproduction, and cellular responses (Brown and Howard, 1985; Brown, 1997). One of the most familiar mechanisms for reacting to deleterious conditions is that of a cellular stress response that involves the rapid synthesis of a set of heat shock proteins (HSPs) (Lindquist, 1986). These proteins act to protect the organism from cell protein damage due to exposure to a wide variety of stressors, including elevated temperature (Lindquist, 1986; Sanders, 1993), cold shock (Nunamaker et al., 1996), oxidative stress (Lindquist, 1986), increased UV irradiation (Sanders, 1993), extreme pH (Koziol

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et al., 1996), osmotic stress (Burg et al., 1996), heavy metals (Köhler et al., 1992; Miller et. al., 1992; Sanders, 1993; Vedel and Depledge, 1995), xenobiotics (Sanders, 1993), and other pollutants (Lindquist, 1986; Blom et al., 1992; Pomerai, 1996; Wiens et al., 1998).

HSPs often include constitutive as well as stress-inducible members. Their role under normal cellular physiological conditions is reflected mostly in regulating protein homeostasis (e.g., degradation of abnormal proteins), in directing the folding and assembly of other proteins, and in transport of proteins within the cell (Lindquist, 1986; Parsell and Lindquist, 1993; Sanders, 1993). Induction of stress proteins (HSPs) has the character of an emergency response (Lindquist, 1986; Parsell and Lindquist, 1993). The increase in synthesis and accumulation of abnormal proteins functions as a trigger for rapid and immediate increase in the expression of HSP genes (Craig and Gross, 1991), with some HSPs remaining at high levels for as long as the stress prevails.

In certain marine habitats, sessile lower invertebrates like sea anemones are directly exposed to extreme environmental conditions, such as elevated temperatures, which may cause tissue damage (Sharp et al., 1994). Stress proteins are expected to play a significant role in conferring tolerance to such harsh conditions upon these organisms.

The Israeli Mediterranean features stressful habitats such as tidal pools. These are very restricted shallow water bodies that sometimes become directly exposed to the air without undergoing any change of water for several hours or days. Consequently, they may be subjected to extreme and unexpected changes in environmental conditions, especially temperature. The Mediterranean also features benign subtidal rocky habitats, characterized by temperate environmental changes.

Owing to the wide exploitation of different ecological zones of rocky shores (tidal pools and subtidal zones) by spatially distinct populations, sessile Cnidaria, such as sea anemones, offer an ideal group of organisms to investigate adaptive responses to changes in environmental conditions.

In this study we examined the ability of the sea anemone *Anemonia viridis,* a zooxanthellate sea anemone abundant along the Israeli Mediterranean shores, to express a 60-kDa HSP (HSP60) as a phenotypic adaptation (acclimatization) to changes in seawater temperature (SWT). This protein is known to play a significant role in resistance to adverse temperatures (Cheng et al., 1989).

Materials and Methods

Seawater Temperature Measurements

Seawater temperatures were constantly measured every 30 minutes by underwater electronic sensors (Optic Stoway,), which were firmly secured in tidal pools and in the subtidal zone. Temperature data were processed by computer software (LogBook for Windows version 2.04), during periods of 1 week before and after seasonal collections of specimens. A portable laboratory thermometer measured temperatures of specific locations in tidal pools every hour (during extreme low tides).

Salinity and pH Measurements

Samples of seawater, which were collected in tubes, were used for salinity measurement with a refractometer (American Optical,) and for pH measurements with a Micro-Processor pH Meter HI 8521 (Hanna Instruments, Italy). The measurements were taken immediately after sampling.

Collection and Maintenance of Animals

Random specimens of *Anemonia viridis* were carefully collected from tidal pools (intertidal zone) at the Sdot-Yam beach and from subtidal zones (2–3 m depth), at the Bat-Yam (near Tel Aviv) and Sdot-Yam beaches (between 31°40′N, 34°32′E and 33°05′N, 35°06′E) during 1998 to 1999. To examine possible effects of low tide on the expression of HSP60, specimens were collected from tidal pools during extreme low tides (October 1998 and October 2000). Some specimens were immediately frozen in a dry-ice container upon collection. Others were transported live in containers with ambient seawater to the laboratory, where they were acclimated in an aquarium with aerated seawater at 23°C, salinity of 39%, and a lighting regimen of 11 hours light and 13 hours dark (100 μmol photons m^{-2} s^{-1}) for several weeks, before exposing them to heat shock experiments.

Stress Experiment

Two groups of specimens (n = 5 for each group) were incubated in 3-L aquaria with filtered seawater (0.22 μ) under continuous aeration and the above-described lighting regimen. One group was subjected to 23°C and the other to 31°C, for 1 week, representing the period of exposure to different subtidal temperatures. All other factors (e.g., 39%

salinity, pH 8.3, irradiance of 100 μmol photons m^{-2} s^{-1}) were identical in both experimental groups. Portions from the tentacles and foot were then cut by scalpel from each sea anemone and frozen immediately in liquid nitrogen.

Protein Extraction

Frozen portions (each approx. 1 g) were ground with a mortar and pestle and immersed in 1 ml of buffer containing 0.5 M NaCl, 100 mM Tris-HCl, pH 7.5, 10 mM EDTA (Bythell et al., 1995) and a cocktail of protease inhibitors consisting of 2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 1.4 μ *trans*-epoxysuccinyl-L-leucylamido (4-guanido) butane (E-64), 130 μM bestatin, 1 μM leupeptin, 0.3 μM aprotinin, and 1 mM sodium EDTA (Sigma, St. Louis, Mo.). Homogenates were centrifuged at 14,000 rpm for 20 minutes at 4°C. Supernatants were frozen at −20°C (the pellet contains the zooxanthellae).

Electrophoresis and Immunoblotting

Aliquots of protein samples (10 μl) were used for protein concentration estimations according to Bradford (1976). Protein samples were dissolved in loading buffer (0.5 M Tris-HCl, pH 6.8, 10% sodium dodecylsulfate [SDS], glycerol, 2-β-m-ethanol, and 0.05% bromophenol blue) and boiled for 2 to 3 minutes before separating. Proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Harlow and Lane, 1988) on 14% gels, loading equal amounts of total protein in each lane. Electrophoresis was conducted at 125 V, 150 mA for approximately 2 hours in XCELL II MINI CELL apparatus (NOVEX,). Proteins were electrophoretically transferred to nitrocellulose membrane by XCELL II blot module (NOVEX), at 25 V, 150 mA for 2 hours. Membranes were stained with Ponceau S to mark the location of the protein bands, following a 1-hour blocking in phosphate-buffered saline (PBS, pH 7.4) containing 0.02% (wt/vol) Tween-20 and 5% skim milk powder.

After washing in PBS-Tween (5 minutes, 3 times), membranes were incubated with monoclonal antibody (IgG mouse clone LK-2) anti-HSP60 at 1:1000 dilution in PBS-Tween, 5% skim milk. The antibodies were purchased from Stressgen (Victoria, B.C., Canada) and are known to identify HSP60 in both eukaryotes and prokaryotes. The epitope is located between residues 383 and 419 of HSP60 (Boog et al., 1992). After washing again in PBS-Tween (5 minutes, 3 times), membranes were incubated with secondary antibody (dilution 1:5000 in PBS-Tween, 5% skim milk), anti-mouse IgG conjugated with horseradish peroxidase (Sigma). Bands were detected by enhanced chemiluminescence (ECL) (Harlow and Lane, 1988). Controls for specificity of the primary antibody were conducted using recombinant human HSP60 free of *Escherichia coli* GroEL (Stressgen) and chicken embryo cells extracts containing HSP60. Control for cross-reactivity with HSP70 was conducted using cell extracts containing HSP70.

Quantification and Analysis of Results

Band densities were detected by ImageMaster 1D densitometer. In order to compare multiple immunoblots and to compensate for variation caused by antibody binding and during the ECL detection, equal amounts of reference protein samples were loaded on each gel. Intensities of the HSP60 bands were normalized relative to the intensity of the reference protein band.

Results

Expression of HSP60 in *Anemonia viridis*

A remarkable expression of HSP60 by the studied sea anemones was recognized by the specific monoclonal antibody (Figure 1). This protein was expressed at high levels

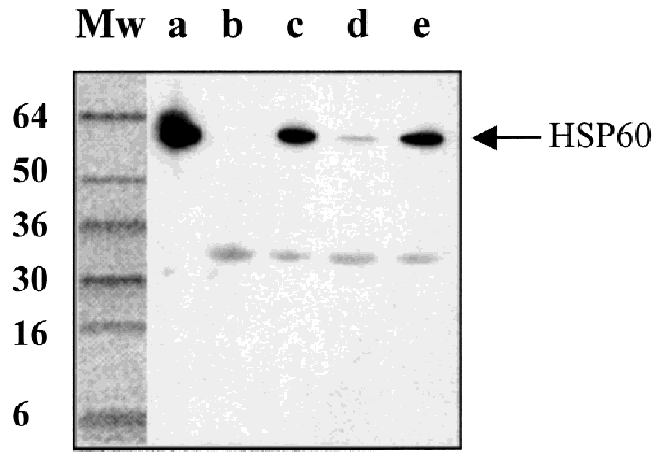

Figure 1. *Anemonia viridis.* Identification of HSP60 by Western blot analysis. Specimens were incubated at 23°C in the laboratory (lanes b and d), exposed to 31°C heat shock in the field (lane c), and removed during extreme low tide in a tidal pool on April 1998 (lane e). Chicken embryo cell protein extraction containing HSP60 was used as a positive control (lane a). Molecular weight standards (lane Mw) are in kilodaltons.

under elevated SWT (31°C during August 1998) and during extreme low tide, when habitat conditions were presumably harmful to the organisms. It was expressed at a constitutive low level under normal laboratory conditions (23°C). No cross-reactivity was found with HSP70 extracts. The appearance of a 33-kDa low-density band in all samples is an often-encountered unspecific binding of the antibody (Sigma).

Differential Expression of HSP60 in *Anemonia viridis* from Tidal Pools

Anemonia viridis that colonize tidal pools may occasionally experience the generation of temperature layers in the tidal pools, separated by a thermocline. This situation occurs on the rare occasions that tidal pools are disconnected from the open sea for 24 hours to several days. Figure 2 offers a schematic presentation of such a tidal pool, colonized by a large number of sea anemones (approx. 150/m^2), presumably from the same genetic clone. When temperature layers appear, different specimens from the same tidal pool are simultaneously exposed to different temperatures. During such extraordinary events in October 1998 and in October 2000, we collected samples from 2 temperature layers (28°C and 23°C, Figure 2) and examined their levels of HSP60 expression. Figure 3 shows that sea anemones exposed to the warmer layer of water (28°C) expressed significantly higher levels of HSP60 than those that were 30 cm deeper and thus exposed to the colder layer (23°C) (Kruskal-Wallis test, $P < .05$). Measurements of SWT and long-term observations over several weeks showed that there had been no extreme low tides and SWT fluctuations before this event (data not shown). Measurements of salinity and pH showed negligible differences between the warmer upper layer and the colder lower layer.

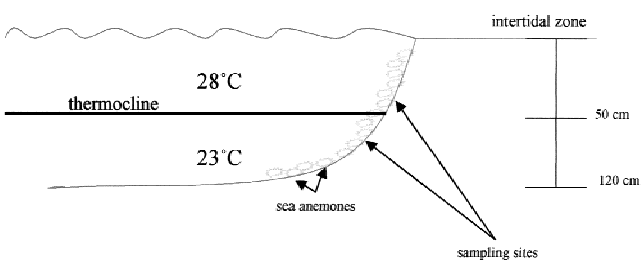

Figure 2. Schematic illustration of the thermocline in a tidal pool during the October (1998 and 2000) extreme low tides.

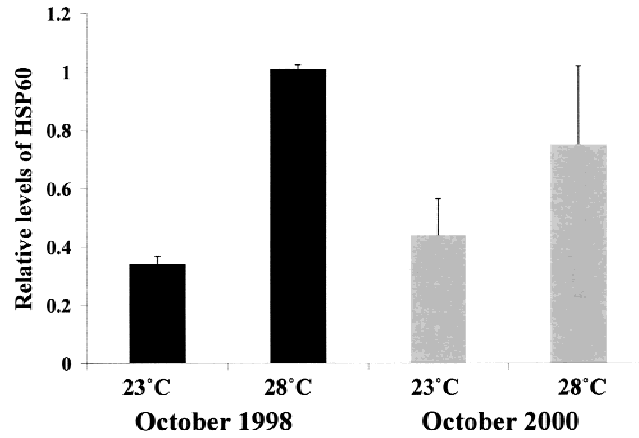

Figure 3. *Anemonia viridis.* Expression of HSP60 in specimens from different depths of the same tidal pool in October 1998 (mean + SD, $n = 2$) and October 2000 (mean + SD, $n = 9$). Specimens were removed from 0.1–0.5-m depth (28°C) and from 0.5–1.2-m depth (23°C) during extreme and uncommon low tide.

Response of *Anemonia viridis* to Seasonal Changes in Seawater Temperature

Differential expression of HSP60 was found in sea anemones according to seasonal changes in subtidal SWT (18°–31°C) along the Israeli Mediterranean coast. The results are shown in Figure 4. A highly significant level of HSP60 was recorded in specimens collected during August 1998, when the subtidal SWT reached 31°C, compared with the levels of expression when SWT ranged between 18°C and 25°C (Kruskal-Wallis test, $P < .005$). Salinity and pH measurements during each seasonal sampling showed no significant deviations between seasons (results not shown).

Laboratory Experiments

Sea anemones were maintained at 23°C and 31°C for 1 week. Figure 5 shows high levels of HSP60 in specimens subjected to 31°C. A lower constitutive level of HSP60 can be seen in specimens that were maintained at 23°C for the same period.

Discussion

The heat shock response acts to protect organisms from possible cell protein damage resulting from a wide variety of stressors including extreme temperature, osmotic stress, UV

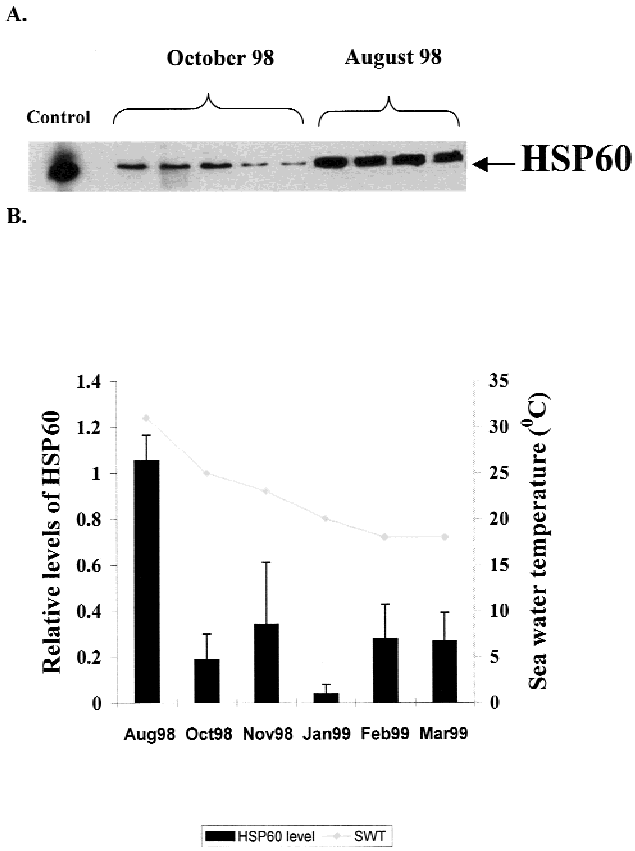

Figure 4. *Anemonia viridis.* Seasonal changes in HSP60 expression in subtidal sea anemones. **A:** Western blot analysis of HSP60 expression: comparison between August 1998 and October 1998 (control is chicken protein extraction, which contains HSP60). **B:** Relative levels of HSP60 expression (mean + SD, August 1998, $n = 4$; October 1998, $n = 7$; November 1998, $n = 6$; January 1999, $n = 6$; February 1999, $n = 8$; March 1999, $n = 9$), accompanied with SWT.

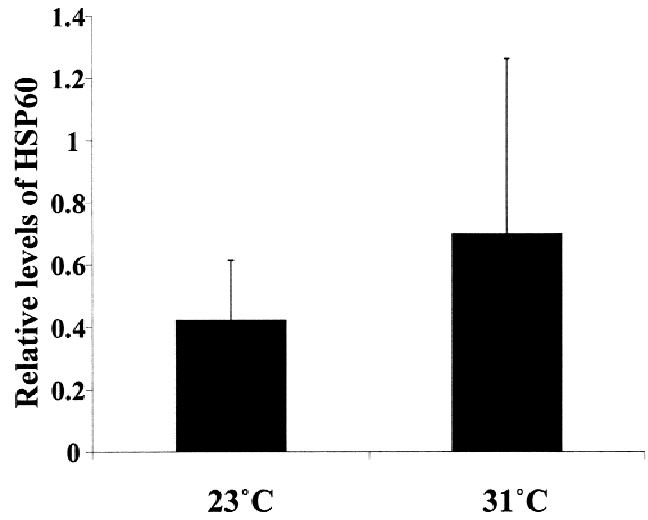

Figure 5. *Anemonia viridis.* The influence of temperature on the expression of HSP60 (mean + SD, $n = 5$) under laboratory conditions. Specimens were incubated at 23°C or 31°C for 1 week.

radiation, and heavy metals. The response entails the rapid synthesis of a set of HSPs that enable the cell to withstand denaturative conditions. The ability to produce this response is thus expected to play a major role in organisms that inhabit stressful environments.

We report for the first time on the use of immunological methods to study expression of HSP60 in sea anemones. Our results show an acclimatization of the sea anemone *Anemonia viridis* to SWT changes in natural habitats, by differential expression of HSP60.

Experimental evidence that HSPs are involved in conferring tolerance to environmental extremes, such as elevated temperature, is abundant in both aquatic and nonaquatic species including Anthozoans (Sanders, 1993; Sharp et al., 1994, 1997; Black et al., 1995; Hayes and King, 1995; Fang et al., 1997; Tom et al., 1999). Many studies have demonstrated HSP70 and low molecular weight HSPs as the major HSPs induced as a result of raised temperature. However, Bosch et al. (1988), using metabolic radiolabeling methods, showed an induction of HSP60 in *Hydra* species and correlated it to the acquisition of thermotolerance by the organism. Later studies by the same author (Gellner et al., 1992) claimed that this protein might be HSP70 and not HSP60.

HSP60 is a member of the chaperone family, known to bind target proteins in order to facilitate folding and assembly under normal conditions. Under adverse temperatures, the production of damaged proteins increases and causes a subsequent induction of HSPs such as chaperones (Langer and Neupert, 1991; Welch, 1993). The results of the present study suggest that elevation of HSP60 levels is a phenotypic adaptation (acclimatization) of sea anemones to stressful SWT. This is well supported by our findings related to the heat shock response of sea anemones during broad seasonal changes in subtidal SWT (Figure 4). It seems likely that 31°C is a stressful temperature for the sea anemones, necessitating a high expression of HSP60 for long periods (days to several weeks) for them to survive. The lower constitutive levels of HSP60 that occur at much less stressful temperatures (18°–25°C) may indicate that this HSP60 also has an important function under normal physiological conditions of the organism. During 1 week before and after each sampling time, SWTs (Figure 4) were almost constant, without any fluctuations that might have affected the ex-

pression of HSPs. Hofmann and Somero (1995) showed seasonal patterns of HSP70 and ubiquitin expression for acclimatization in the mussel *Mytilus edulis.* Additional examples of seasonal expression of HSPs are known from other organisms (Dietz and Somero, 1992). However, to the best of our knowledge, this is the first report of seasonal expression of HSPs in benthic marine Anthozoans.

The differential responses of sea anemones (Figure 3) that were 30 cm apart on either side of the thermocline in the tidal pool (Figure 2), during the October 1998 and 2000 low tides, further suggest that HSP60 expression is a process of acclimatization to elevated SWT. Unfortunately, we were able to analyze only 2 specimens in each experiment of October 1998, but 9 in the October 2000 samples. The overall results (Figure 3) strengthen the assumption that *A. viridis* can acclimatize to different temperatures under different natural SWTs. The results of all field experiments show that immediate and unexpected changes in ambient SWT, as well as long-term seasonal changes in SWT, can affect the pattern of HSP60 expression.

Changes in the level of HSP60 in *A. viridis* could be also influenced by other extreme fluctuations in natural abiotic parameters. In a controlled laboratory experiment, we demonstrated that by changing the temperature factor alone, we could affect the heat shock response. However, the laboratory differences in the relative levels of HSP60 between 23°C and 31°C (Figure 5), although substantially higher in 31°C, did not show statistical significance (Kruskal-Wallis test, $P > .05$). The fact that differential expression of HSP60 also could be due to extreme salinity or pH is not probable because only negligible changes were detected in these parameters. The possibility that seawater pollutants affected the expression of this HSP60 is also unlikely because the collection sites are known to be pollution-free (this is a planned site for a nature reserve). It is possible, however, that other extreme abiotic factors prevalent in the natural habitat, (e.g., UV light, dissolved O_2) synergistically affected the expression of HSP60 in *A. viridis.* Hence the observed high expression of HSP60 in *A. viridis* in the field is the outcome of a combination of stressors, of which increased SWT is a major component. The influence of other such factors on the expression of this protein is currently under examination. Future experiments will also investigate the kinetics of the HSP60 response to extreme temperature and to severe short-term fluctuations in SWT.

Another issue that has remained unstudied to date is the possibility that bacteria associated with marine invertebrates are the source of the heat shock proteins found in extracts from marine organisms. Accordingly, during some of the laboratory experiments, we applied antibiotics (penicillin G and kanamycin) to prevent bacterial protein synthesis. No significant differences in the levels of HSP60 were found between groups exposed to antibiotics and those not exposed (results not shown). Furthermore, we used monoclonal antibody anti-HSP60 specific to bacteria, which did not identify HSP60 in *A. viridis* (results not shown). Other organisms, such as the symbiotic algae (*zooxanthellae*), were separated from the animal tissue during the extraction process (Bythell et al., 1995), and thus their proteins are not included in the final extraction. Currently, we are deciphering the HSP60 gene sequence of *A. viridis,* which will present the most reliable answer to the biological origin of this protein.

This study may offer a useful tool for detecting HSP60 in marine invertebrates. We have shown that this method enables monitoring of differences in HSP60 expression under natural conditions, which may be used as an indication of stress due to changes in SWT. The results contribute to the understanding of the role of HSPs in marine invertebrate phenotypic adaptation to stressful habitats, which may have implications for their abundance and distribution in various habitats.

The expansion of this study to other marine organisms such as stony corals is of great importance, considering that SWT in many tropical regions is projected to increase by 1° to 2°C per century (Guldberg, 1999). The worldwide degradation of coral reefs due to global climate change and human perturbations requires the development of an early warning system for ambient assessment of the health of marine benthic communities (Smith and Buddemeier, 1992). This research offers the use of HSP60 as a possible biomonitor, in addition to other HSPs such as HSP70 (Koziol et al., 1996; Pyza et al., 1997), that will enable assessment of the effect of environmental changes on marine organisms. In order to further develop such a system based on HSP60 expression, it is necessary to investigate the relations between the specific HSP60 induction, the kinetics of the HSP60 response, the type of stress, other physiological and behavioral responses to stress by sessile marine organisms, and consequent effects on marine benthic communities and ecosystems. Characterization of HSP60 responses as well as other HSPs in marine invertebrates may help to predict their ability to survive future possible short-term and long-term changes in SWT.

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